

p16/CDKN2 alterations and pRb expression in oesophageal squamous carcinoma

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Abstract

Background—Upregulation of the cell cycle associated genes, p16/CDKN2 and the retinoblastoma susceptibility gene (Rb), is commonly seen during the proliferation of normal cells. An inverse relation between the expression of p16/CDKN2 and Rb has been noted in many tumours, but has not yet been determined in oesophageal squamous carcinoma.

Aims—To investigate p16/CDKN2 genetic alterations and both the p16/CDKN2 and the Rb protein (pRb) immunophenotypes in oesophageal squamous carcinoma.

Methods—Twenty primary oesophageal squamous carcinomas were examined for mutations in p16/CDKN2 by the polymerase chain reaction, single stranded conformational polymorphism, and DNA sequencing. Synthesis of p16/CDKN2 and pRb proteins was determined by immunohistochemistry in 19 specimens of formalin fixed, paraffin wax embedded tissues.

Results—Mutations of p16/CDKN2 were not detected in exons 1 and 2. In only one case, G to C and C to T base changes were detected in a non-coding region of exon 3. Expression of p16/CDKN2 and Rb was observed in both normal and neoplastic areas of tissue sections, indicating neither consistent homozygous deletion nor consistent hypermethylation of the genes in tumours. Fourteen tumours showed an inverse expression of p16/CDKN2 and Rb. An increased percentage of cells that immunostained positively for p16/CDKN2 but not for pRb was observed in eight tumours, five of which had no detectable pRb, suggesting defective Rb expression in these oesophageal squamous carcinomas. **Conclusions**—These results indicate that p16/CDKN2 mutations occur infrequently in oesophageal squamous carcinoma. The alteration of the Rb gene is suggested as an important step in the development of these tumours.

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Keywords: pRb; p16/CDKN2; oesophageal cancer; oesophageal tumorigenesis

The retinoblastoma susceptibility gene product (pRb) is involved in cell cycle regulation and functional pRb acts as a suppressor, inhibiting the entry of cells from G₁ into the S phase.¹ An upstream regulator for pRb has been identified as the p16/CDKN2 protein.² It is a cyclin dependent kinase (CDK) inhibitor and prevents wild-type pRb from inactivation by CDK

catalysed hyperphosphorylation.^{3,4} Alteration of either pRb or p16/CDKN2 may allow cells to enter the S phase of the cell cycle, thereby gaining a growth advantage.

The significance of p16/CDKN2 intragenic mutations in development of oesophageal squamous carcinoma is controversial because reported frequencies are highly variable among studies.⁵⁻⁹ Differences have been suggested to be due to geographical variation in genetic and/or environmental factors.^{6,9} Regardless of the variation, the most common mechanism for the inactivation of p16/CDKN2 appears to be homozygous deletion.¹⁰ The alteration has been observed frequently in oesophageal squamous carcinoma cell lines.^{11,12}

Compared with cancer cell lines, alterations of p16/CDKN2 are generally rare in primary tumours.¹⁰ It has been suggested that the high frequency of p16/CDKN2 alterations in cancer cell lines is a culture artefact.¹³ However, false negativity as a result of contamination by non-neoplastic stromal cells is also possible, and may contribute to the rarity of p16/CDKN2 alterations. To overcome the effect of such dilution by stromal cells, an immunohistochemical technique has been used to examine alterations in p16/CDKN2.¹⁴ Lack of p16/CDKN2 expression indicates either homozygous deletion or hypermethylation at the 5' CpG island of the gene, which also occurs in many human tumours.^{6,15,16}

An inverse relation between p16/CDKN2 and Rb expression has been observed in many human primary neoplasms and cancer lines.^{6,17-19} In particular, pRb deficient cells tend to synthesise large amounts of p16/CDKN2 protein.²⁰⁻²² Few studies have examined the expression of Rb in oesophageal carcinomas.^{23,24} The role of pRb and its possible relation to p16/CDKN2 in the regulation of oesophageal squamous tumorigenesis remain unclear and need to be established.

We examined the p16/CDKN2 gene for mutations and both the p16/CDKN2 and Rb genes for expression in oesophageal squamous carcinoma in northeast Italy, where this tumour has been associated with tobacco and alcohol consumption. The relation of p16/CDKN2 and Rb expression was determined for each tumour. The roles of p16/CDKN2 and pRb in oesophageal squamous tumorigenesis are also discussed.

Methods and materials

SAMPLE PREPARATION

Surgical samples from 20 patients (20 men; mean age 54 years, range 48-69) who had undergone oesophagectomy for invasive squamous carcinoma were obtained from the

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Table 1 Primers for the amplification of 16/CDKN2 exons

		Primers	
	Size (bp)	Upstream	Downstream
Exon 1	164	5'-GGGGAGCAGCATGGAGCCTT-3'	5'-CTGCTCCCGCTGCAGACCCT-3'
Exon 2A	166	5'-GGCTCTGACCAATTCTGTTCTCTCT-3'	5'-ACCACCAGCGTGTCCAGGAA-3'
Exon 2B	163	5'-CTACCCGACCCGTGCACGA-3'	5'-CGCGCAGGTACCGTGCAGACA-3'
Exon 2C	161	5'-CCCGTGGACCTGGCTGAGGA-3'	5'-TGGAGCTCTCAGGGTACAAATTCT-3'
Exon 3	174	5'-CCTGTTTTCTTTCTGCCCTCT-3'	5'-TTTAAAGCTCTATTTTCTAAATGA-3'

department of pathology, University of Padova, Italy. Fresh samples of normal and malignant tissues were collected for DNA extraction and were stored at -80°C after histological examination (cryostat sections). Tissue samples adjacent to the specimens collected for molecular analysis were fixed in 10% buffered formalin and embedded in paraffin wax for histological and immunocytochemical examination. The histological diagnosis of oesophageal squamous carcinoma was based on standardised criteria and the tumour stage was assessed by means of the international TNM system.²⁵

DNA EXTRACTION, PCR-SSCP ANALYSIS, AND DNA SEQUENCING

Fresh oesophageal tissues were digested with proteinase K and high molecular weight genomic DNA was extracted and purified by phenol/chloroform methods as described previously.²⁶ Exons 1–3 of the p16/CDKN2 gene were amplified by the polymerase chain reaction (PCR) using five primer sets (E1, E2A, E2B, E2C, and E3), as shown in table 1. PCR was performed in a 25 μl reaction solution containing 50–100 ng of genomic DNA, 200 μM deoxyribonucleoside triphosphates, 1.5 mM MgCl_2 , 0.5 μM primers, 2.5% dimethylsulphoxide, 1 \times PCR buffer (20 mM $(\text{NH}_4)_2\text{SO}_4$, 0.01% (wt/vol) Tween, 75 mM Tris-HCl, pH 9.0), and 1 U thermostable DNA polymerase (Advance Biotechnologies, Columbia, Maryland, USA). Thermal cycling was carried out at 94°C for 30 seconds, 63°C (E1, E2A, E2B, and E2C), or 55°C (E3) for 30 seconds, and 72°C for one minute in a total reaction of 40 cycles after initial denaturation for five minutes at 94°C . A negative control containing no DNA template was run in parallel for each amplification.

Single stranded conformational polymorphism (SSCP) analysis for gene mutations was modified from a previous report.²⁷ Aliquots of

1 μl of PCR products were denatured in 5 μl loading buffer (95% formamide, 20 mM disodium EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue) at 95°C for five minutes and then quickly plunged into ice to prevent renaturation. These denatured PCR products (6 μl) were loaded on to a 12% non-denaturing polyacrylamide gel with 5% glycerol. All SSCP reactions were carried out at room temperature for four to five hours. Electrophoresis was performed at a constant 10 mA for E1, E2A, E2B, and E2C fragments, or at a constant 100 V for E3 PCR products. Single stranded DNA was visualised by silver staining according to the manufacturer's instructions (Bio-Rad, Richmond, California, USA).

Samples with a mobility shift were verified by a second, independent PCR-SSCP. DNA sequencing using Sanger's enzymatic method and an automatic sequencer to resolve sequencing "ladders" was performed by a commercial laboratory (Primm SRL, Milano, Italy).

IMMUNOHISTOCHEMISTRY

Dewaxed 5 μm sections were immersed in a citrate buffer solution (0.01 M sodium citrate, pH 6.0) and boiled in a microwave oven at 600 W for 10 minutes. Immunostaining for pRb was performed using the monoclonal antibody IF8 (NeoMarkers, Fremont, California, USA). A polyclonal antibody, C-20 (Santa Cruz Biotechnology, Santa Cruz, California, USA) was used for p16/CDKN2 immunohistochemistry. The immunohistochemical reaction was detected by an avidin-biotin conjugation kit, according to the manufacturer's instructions (Vector Laboratories, Burlingame, California, USA). In short, after blocking endogenous peroxidase activity with 0.3% H_2O_2 , the sections were incubated overnight at 4°C with either the anti-pRb or anti-p16/CDKN2 antibody at a working dilution of 1/100. Sections were then treated with a biotinylated secondary antibody for 30 minutes, followed by avidin-biotin peroxidase conjugate and a colour reaction with 3,3'-diaminobenzidine substrate (Sigma Chemical, St Louis, Missouri, USA). Negative controls were established by replacing the primary antibody with PBS and normal mouse serum. Known positive control sections for both p16/CDKN2 (small cell lung cancer) and pRb (gastric cancer) were included in each batch of immunostained specimens.

Only nuclear immunoreactivity was considered to be a genuine indication of the presence of the pRb or p16/CDKN2 proteins. The nuclear immunoreaction for pRb and p16/CDKN2 was semiquantitatively scored in both the normal and the neoplastic areas of two

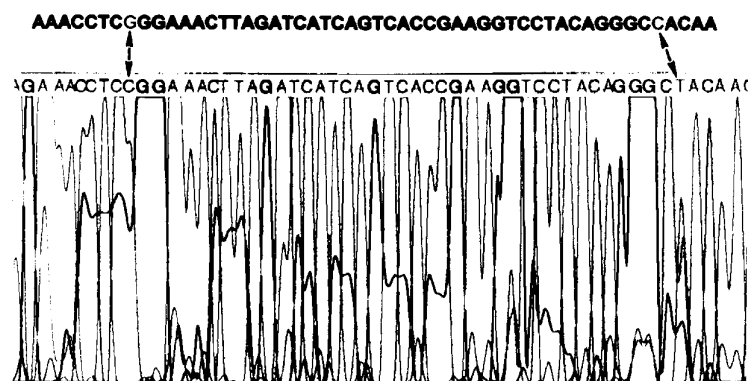


Figure 1 DNA sequencing analysis of mutation for p16/CDKN2 in exon 3 (case 8).

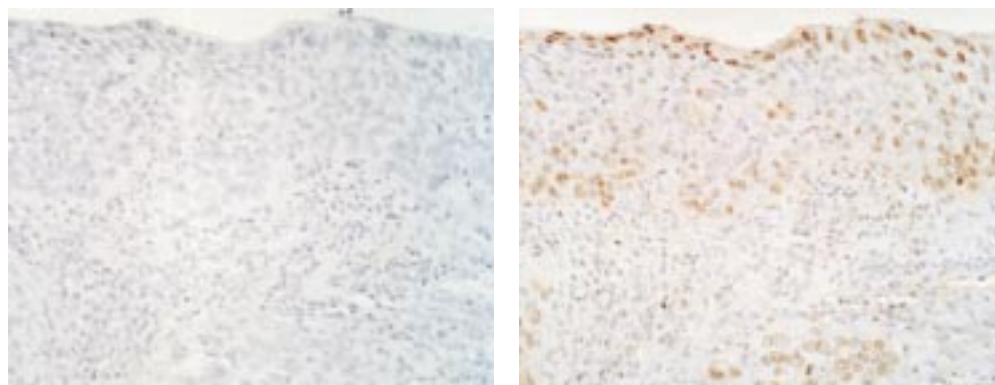


Figure 2 Oesophageal squamous cancer (case 4). (Left) Lack of nuclear staining for the pRb antigen (avidin–biotin complex method). (Right) Nuclear immunostaining for the p16/CDKN2 protein seen throughout the neoplastic epithelium (original magnification, $\times 175$).

serial sections. A mean percentage of immunostained nuclei was obtained from 10 high power microscopic fields chosen at random. Three levels of nuclear immunostaining were distinguished, as follows: G0, absence of any nuclear immunostaining; G1 (low grade immunoreaction), nuclear immunostaining not exceeding 5% of the tested areas; G2 (high grade immunoreaction), positive nuclear immunostaining in more than 5% of the nuclei of the tested areas (no cases scored as G2 showed a percentage of positive nuclei lower than 25%; range 25–75%).

Results

Examination of exons 1–3 of the p16/CDKN2 gene in 20 oesophageal squamous carcinomas did not reveal any mutations in coding regions of the gene. In only one case (case 8), base changes were detected in the non-coding region of exon 3, with a G to C transversion and a C to T transition at the 29th and the 69th nucleotide downstream of the stop codon, respectively (fig 1). The base changes were confirmed by repeat, independent PCR-SSCP and DNA sequencing.

Nuclear immunoreactivity was always detected in normal oesophageal tissue and it was consistently restricted to the basal/parabasal layers (proliferative zone) of the squamous epithelium (score level, G1). As for the neoplastic areas, the percentages of nuclear immunostaining for p16/CDKN2 and pRb varied among tumours (table 2). Comparing malignant and normal tissues, neoplastic nuclei showed a higher percentage of immunoreaction for p16/CDKN2 in eight cases (cases 4, 5, 7, 9, 10, 14, 17, and 19). In three such cases, pRb concentrations were in the same range (G1) in normal and neoplastic areas, whereas in the remaining five of eight cases (cases 4, 5, 7, 10, 19) pRb was undetectable in the malignant tissue. A representative case exhibiting a high grade (G2) immunoreaction for p16/CDKN2 in the absence of any positive immunoreaction for pRb (G0) is shown in fig 2. High grade (G2) expression of Rb was seen in six tumours (cases 6, 8, 11, 12, 18, and 20), and in all of these cases it was associated with a low grade expression of p16/CDKN2. Loss of p16/CDKN2 expression was not detected in any tumours.

Table 2 Mutations of p16/CDKN2 gene and protein expression of p16/CDKN2 and Rb

Case	p16/CDKN2 mutations	p16/CDKN2 IHC		pRb IHC		Tumour stage
		Normal	Tumour	Normal	Tumour	
1	—	G1	G1	G1	G1	IIB
2	—	G1	G1	G1	G1	IIA
3	—	G1	G1	G1	G1	IIB
4	—	G1	G2	G1	G0	III
5	—	G1	G2	G1	G0	III
6	—	G1	G1	G1	G2	IIA
7	—	G1	G2	G1	G0	IIA
8	G to C and C to T*	G1	G1	G1	G2	IIB
9	—	G1	G2	G1	G1	IIB
10	—	G1	G2	G1	G0	IIA
11	—	G1	G1	G1	G2	IIA
12	—	G1	G1	G1	G2	IIA
13	—	G1	G1	G1	G1	III
14	—	G1	G2	G1	G1	IIA
15	—	NT	NT	NT	NT	IIB
16	—	G1	G1	G1	G1	Unknown
17	—	G1	G2	G1	G1	III
18	—	G1	G1	G1	G2	IIA
19	—	G1	G2	G1	G0	IIB
20	—	G1	G1	G1	G2	IIB

G0, no detectable nuclear immunostaining; G1, no more than 5% nuclear immunostaining; G2, positive immunostaining in more than 5% of the nuclei (no cases scored as G2 showed positive immunostaining in less than 25% of the neoplastic nuclei; range 25–75%).

*, Base changes located at the 3' non-coding region of exon 3.

IHC, immunohistochemical staining; NT, not tested; —, negative.

Discussion

A low frequency of p16/CDKN2 intragenic mutations has been reported previously in primary oesophageal squamous carcinoma, and our finding of no mutation in the coding region of p16/CDKN2 is consistent with a report in an Italian series selected from the same high risk geographical area (world standardised rate for oesophageal squamous carcinoma in men: 9.7).⁹ To our knowledge, base changes in the non-coding region of exon 3 (case 8) have never been reported in the literature. Their biological relevance to tumorigenesis remains to be determined.

The pattern of sporadic immunostaining for p16/CDKN2 and pRb in normal and neoplastic cells has been observed in previous studies.^{14–28} Cells with positive nuclear immunostaining might be undergoing proliferation, because an increase in p16/CDKN2 and Rb expression has been observed in cells progressing to the S phase of the cell cycle.^{20–29} In normal oesophageal epithelium, the presence of p16/CDKN2 and pRb immunostaining mainly in basal/parabasal cell layers (which is considered to be the proliferative compartment) supports the notion that upregulation of p16/CDKN2 and Rb is associated with cell proliferation.

Detection of p16/CDKN2 expression in both the normal and neoplastic tissues of 19 oesophageal squamous carcinoma samples examined, regardless of tumour stage, proves that neither consistent homozygous deletion nor consistent hypermethylation of the p16/CDKN2 gene has occurred. In the present study, p16/CDKN2 was detected immunohistochemically in only a proportion of the neoplastic nuclei. One may wonder whether such an immunostaining pattern represents the phenotypic counterpart of the genotypic heterogeneity of the cancer cell. It is conceivable that the positive immunoreactivity restricted to a proportion of the neoplastic nuclei could be ascribed to the sensitivity of the method used, which would be able only to detect accumulation of the gene product in active, replicating cells. This would be in keeping with the finding of positively stained nuclei in the proliferative zone of the benign squamous cell epithelium. However, a high frequency of negative nuclear immunostaining for p16/CDKN2 and the occurrence of homozygous deletion or hypermethylation of the p16/CDKN2 gene have been reported recently in oesophageal squamous carcinomas collected in China and in Japan, respectively.^{30–31} The difference in frequency of these types of p16/CDKN2 alteration in our study compared with those mentioned above, as seen in intragenic mutations,^{5–9} suggests that the mechanism of oesophageal squamous carcinoma tumorigenesis may differ according to the geographical area.

In contrast to the consistent numbers of cells with detectable p16/CDKN2 and Rb expression in normal tissues, the percentage of such cells varies among tumours. The pattern of expression involves an inverse association between p16/CDKN2 and Rb in most oesophageal tumours (14 of 19), consistent with the findings

in other tumour types.^{6–17–19} The inverse relation has been demonstrated by *in vitro* studies, in which a marked increase in the p16/CDKN2 protein has been observed in pRb deficient cells or cells transformed with oncogenic viruses.^{20–22} In our study, the absence of Rb expression accompanying an increase in p16/CDKN2 expression in five tumours (cases 4, 5, 7, 10, and 19) is consistent with the mechanism of pRb deficiency observed in *in vitro* systems. For three further tumours (cases 9, 14, and 17), the manifestation of an upregulation of p16/CDKN2 but not of Rb suggests a possible pRb deficiency, although Rb expression is not completely lost. It has been shown that immunohistochemical analysis cannot differentiate truncated, mutated, or wild-type pRb.³² Direct examination of the Rb gene may provide evidence of truncated or mutated pRb. However, screening for Rb gene mutations or deletions is often hampered by the size of the gene, which contains 27 exons, spanning a length of about 200 kilobases.³³ Although increased p16/CDKN2 expression may also result from loss of wild-type pRb function by forming a complex with the E7 protein of human papillomavirus, our previous failure to detect human papillomavirus in oesophageal squamous carcinoma from the same geographical area does not support the idea of this mechanism in pRb inactivation in the current tumour series.³⁴

Downregulation of p16/CDKN2 expression by wild-type but not deficient pRb has been reported in non-transformed cell lines.²¹ Our observation of an increase in Rb expression but not in p16/CDKN2 expression (cases 6, 8, 11, 12, 18, and 20) suggests that wild-type pRb is suppressing the increase in p16/CDKN2 expression. In this circumstance, the mechanism of oesophageal squamous carcinoma tumorigenesis might involve phosphorylation of pRb. However, this hypothesis remains to be tested for the pRb protein. The remaining tumours (cases 1, 2, 3, 13, and 16) showed no inverse relation between p16/CDKN2 and Rb expression and, therefore, failed to provide any indication of the role of p16/CDKN2 or pRb in oesophageal squamous carcinoma tumorigenesis.

In short, we have detected a low frequency of p16/CDKN2 gene alterations in oesophageal squamous carcinoma from northeast Italy. This finding is consistent with other Italian series, but differs from reports from other geographical areas, notably Japan. The inverse expression of p16/CDKN2 and Rb in oesophageal squamous carcinoma is consistent with findings for other tumours. Furthermore, aberration of pRb appears to be an important step in the development of oesophageal squamous carcinoma.

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